Animal Health Research Journal Vol. 7, No. 1, March 2019 pp. 227-237

ISSN: 2356-7767

Validation of HPLC methods for detection of selenium and Nano-selenium in chicken tissues

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Received in 12 /2/2019
Accepted in 19/3/2019

Abstract

As a part of an ongoing study to develop and validate an accurate, sensitive and reproducible reverse phase high performance liquid chromatographic (RP-HPLC) method with a UV detector for the quantization of selenium (Se) and nano-selenium (N-Se) in chicken tissues (muscle and liver) following oral supplementation of Se and N-Se at a dose 100mg/L. Pre-column derivatization and reversed-phase chromatography provides separation of nano-selenium compound and eluted from a C8 column (4.6 mm i.d.,150 mm, 5µm particle size, pore size100 Å) and the mobile phase was methanol (85%):10mM citric acid monohydrate (15%) with adjusted pH of citrate buffer at 2.5.The samples were extracted for selenium detection by perchloric and nitric acids. Separation of selenium compound was carried out onC18 column (4.6 mm, i.d., 250 mm, 5 µm) with a mobile phase 100% methanol. A UV detector set at 332 nm and 280nm were used to monitor the effluent (nano-selenium and selenium), respectively. The limit of detection and quantification was found to be 0.014032 µg/ml,0.046775µg/ml and 0.008657µg/ml, 0.026234µg/ml for Se and N- Se, respectively. Linearity for N-Se was in a range of 0.01 to 2.5 µg/ml and for Se was 0.005 to 2.5 µg/ml. The proposed methods were highly sensitive, accurate and precise and could be used for routine analysis of N-Se and Se in chicken tissues.

Keywords: *RP, HPLC, Selenium, Nano Selenium, Chicken tissues.*

Introduction

Selenium is a trace element essential in animal nutrition and exerts multiple actions related to animal production, fertility, and disease prevention (Mervyn,1985). Selenium is an integral part of the enzyme glutathione eperoxidase, which serves as an antioxidant enzyme that helps to control levels of hydrogen peroxide and lipid peroxides that are produced during normal metabolic activity **(Rotruck *et al*., 1973).** In addition, dietary selenium is essential for the activity of virtually all arms of the immune system **(Suraiand Dvorska, 2002).** Selenium, like all biologically essential trace elements, can be toxic when provided at levels in excess of the biological requirement. Trace elements typically demonstrate what has been referred to in toxicology as a Ushaped response curve **(Hayes, 2008),** which describes the negative effects of selenium deficiency and the negative effects of selenium at excessive dietary inclusion levels. Selenium has long been known to be toxic and there are concerns about its effect on animals and animal products. To ensure feed safety, maximum levels for selenium in complete feeds have been set at 0.5 mg/ kg in the European Union (2004) and China **(Ministry of Agriculture, 2010)** and 2.0 mg/

kg for the United States **(AAFCO, 2011).**

Nano-materials exhibit novel properties, such as great specific surface area, high surface activity, a lot of surface active centers and high catalytic efficiency **(Gao and Hiroshi, 2005).** Due to the advantage of size effect and high surface reactivity, nanoparticle has been already used in pharmaceutical applications to increase the bioavailability of drugs and targeting therapeutic agents to particular organs **(Florance *et al.*, 1995; Davda and Labhasetwar, 2002).** Nano red elemental selenium (nano- Se) of size 5–100 nm was synthesized by **(Zhang *et al.*2001)** and observed that nano -Se had a similar bioavailability in rat and much less acute toxicity in mice compared with selenite. It has been reported that nanoparticle showed new characteristics of transport and uptake and exhibited higher absorption efficiencies **(Davda and Labhasetwar, 2002; Chithrani and Chan, 2007; Zha *et al.*, 2008; Liao *et al.*, 2010).** Advantages of ultrasound-assisted extraction of trace elements from a variety of matrices using both bath and probe ultrasonic processors technique have been widely recognized for efficient separation of metals and metalloids from a variety of solid matrices, hence avoiding intensive and destructive sample treatments such as dry ashing or acid digestion procedures. Ultrasonic probes provide some advantages in comparison with ultrasonic baths, namely, short extraction times, extraction efficiency approaching 100% and feasibility of using low acidic conditions for extraction as a result of the enhanced ultrasonic power delivered by the former (i.e., high intensity sonication), which can be directly focused onto the sample (Bendicho & Lavilla, 2000). The aim of this study is to develop and validate new methods for extraction and quantification of selenium and its Nano form by VIS-UV RP-HPLC in chicken tissues. These methods enabled the quantification of selenium and its nano form residues and their withdrawal time to be safe for human consumption to avoid selenium toxicity.

Materials and methods

a-Instrumentation:

The high-performance liquid chromatography system consisted of Agilent Series 1200 quaternary pump, Series 1200 auto sampler, Series 1200 UV Vis detector and HPLC 2D Chemstation Software (Hewlett-Packard, Les Ulis, Germany).

The separations were performed on reversed-phase (RP) column C18 (4.6 mm, i.d., 250 mm, 5 µm), (Kromasil Co.) and (RP) column C8 (5 µm particle size, pore size 100 Å, L × I.D. 150 mm × 4.6 mm

Cooling centrifuge, ultrasonic bath (Buhler, Germany), vortex mixer (Inc., N.Y., USA) and shaker (Vibra).

b-Reagents, standards and mobile phase:

All commercial chemicals were HPLC grade. Selenium dioxide powder (SeO2) 1000 mg/L, Titrisol, 9915) from Merck. Selenium Nan powder with a particle size (50nm) (NS613010 -171) from Nanoshel.

Methanol, Hexane and Toluene were of HPLC grade, while other chemicals and reagents analytical grade were obtained from BDH Laboratories Supplies (BDH Chemical Ltd., Poole, U.K.).

Deionized, Milli Q water (Millipore, Bedford, MA, USA) was used to prepare the mobile phase and diluent solutions.

c-Chromatographic separation:

Isocratic mobile phase for selenium separation was 100% MeoH (HPLCgrade) and for Nano-selenium separation consisted ofMethanol (85%),10mM citric acid monohydrate (15%) with adjusted pH of citrate buffer at 2.5.

d-Standard preparation:

A stock standard solution of 1 mg/ml was prepared by dissolving 10mg of selenium standard in 10 ml of diluted nitric acid (2%) and the same amount for nano selenium standard was dissolved in 10 ml de-ionized water.

e-Assay procedure

d-1- Extraction method for nano-selenium:

The homogenized tissue sample(1 g) was weighed in a 50 ml propylene tube and add 4

ml of 10% Trichloroacetic acid (TCA), 1ml of 30%Nitric acid and 20µl concentrated sulfuric acid (H_2SO_4)then vortex for 30 seconds. The samples were heated in water bath at 90^0 C for 20 minutes, then 0.1 g of sodium sulfite was added, the samples were ultra-sonicated for 30 minutes the ncentrifugedat 2000 rpm for 10 minutes. The supernatant was filtrated through an acid washed filter paper (TCA 10%). The filtrate pH was adjusted to 1.0 by adding 0.1NHCland followed by the derivatization steps **(Arain *et al.*, 1999).** DAB (2,3-diaminobenzene) reagent (2ml with 1% 0.1N HCL) was added to the sample filtrate, and the tubes were tightly capped. The contents were mixed well, and the tubes placed in a dark at 30^0C in water bath for 40 min for synthesis of 2,1,3 benzoselenadiazole (BSD). After that, 1ml of toluene was added and vortex for 30 seconds and centrifuged at 2000rpm for 5 minutes. The organic phase was collected and evaporated at 40^0C and the residues eluted by 200µl of methanol: water (50:50 v/v). The HPLC sample injection volume was 10µl at flow rate 1ml/min. with UV detection at 332 nm **(Arain *et al.*, 1999)** and mobile phase wasmethanol:**10mM citric acid monohydrate** (85:15)**with adjusted pH of citrate buffer at 2.5.**

d-2- Extraction method for selenium:

One gram of homogenized tissue sample was weighed in 50 ml falcon tube. Onemilliliterof perchloricacid,1ml of Nitric acid 65% and 50µl concentrated sulfuric acid were added then vortex for 60 seconds. The samples were heated on a hotplate at 100^0 C till the white fumes of perchloric acid were evolved. The sample volume was maintained approximately at 1 ml to prevent the volatilization of selenium. Two milliliter of hexane was added and vortex for 30 seconds and centrifuged at 2000rpm for 5 minutes. The organic phase was collected and evaporated at 40^0C.The dried residues were dissolved by 200 µl of a mixture of methanol:2 propanol(1:1).The HPLC sample injection volume was 50µl at flow rate 1ml/min. with UV detection at 280 nm and mobile phase was methanol (HPLC grade).

Method validation

It is the procedure by which the performance characteristics of the method meet the requirements for the intended analytical application. The proposed method was validated according to the International Conference on Harmonization (ICH) guidelines. **ICH, Q2 (R1), Harmonised tripartite guideline, Validation of analytical procedures: text and methodology International Conference Harmonization ICH, Geneva, Nov 2005.**

System suitability

It is an integral test of many analytical procedures. In chromatography, there are some parameters which are column plates (theoretical plates) for judging column efficiency, peak asymmetry factor (As), tailing factor (T_f) and resolution (Rs)**.** Rs is a measure of the separation quality or in other words; is specified to ensure that closely eluting compounds are resolved from each other and according to the united states pharmacopeia **(USP, 2017).**

Precision

2.A. The intraday precision

Precision is the closeness of agreement among a set of results. The intraday precision (Repeatability) of the assay were measured by analyzing six replicate of spiked tissue sample with selenium (0.02µg/mL) and nano-selenium (0.08µg/mL) on the same day. The percent of relative standard deviation (% RSD) for peak responses was calculated according to **(ICH,2005)**.

2.B. Intermediate precision:

Intermediate precision (within-laboratory variation) determined by estimation of six replicates of spiked tissue samples with selenium (0.02µg/mL) and nano-selenium (0.08µg/mL) daily for 6 times over a period of one week (interday precision). The %RSD for peak responses was calculated according to **(ICH, 2005)**

Linearity and range

Linearity was performed by preparing an eight different concentrations of drug standard at squared correlation coefficient of 0.99 (r^2) according to ICH. Calibration lines of peak area ratios (peak area analyte/peak area internal standard) versus concentration were determined by single level calibration curve (linear regression equation, Y = A X +B), where: Y =

peak area ratio, A = slope, X = concentration and B = intercept.

Specificity:
Solutions of standard and spiked tissue samples were prepared as per the test method and injected into the chromatographic system, the chromatograms were recorded.

Accuracy and Recovery:
The accuracy of an analytical method is the closeness of test results (theoretical value) obtained by method to the assay value. Accuracy must be established across the specified range of the analytical procedure. Accuracy was determined over the range of 50%, 100% and 150% of the sample concentration according to **(ICH, 2005)**.
The accuracy was then calculated as the percentage of analyte recovered by the assay.
% Recovery = [Theoretical value / Assay value] × 100
Accepted criteria of recovery ranged from 85-110%

Robustness:
It was determined by detecting how a method stands up to slight variations in normal operating conditions. The chromatographic conditions change in selenium method were studied. The effect of mobile phase composition was assessed at 93% methanol instead of 100% methanol
The determination of nano-selenium method robustness, small deliberate changes in the chromatographic conditions, such as the effect of mobile phase composition was assessed at methanol: **citrate buffer (87:13 v:v)** and methanol : citrate buffer **(83:17 v:v)** instead of methanol: **citrate buffer (85:15 v:v)** .The effect of pH of citrate buffer of mobile phase at 2.3 and 2.7 instead of 2.5. The effect of column (C18 instead of C8) was studied. The %RSD of robustness testing under these conditions was calculated in all cases.

Limits of detection (LOD)
It is considered to be the quantity yielding a detector response which gives signal to noise ratio 3:1 according to (ICH,2005)

Limits of quantification (LOQ)
It is the lowest amount that can be analyzed within acceptable precision and accuracy which give signal to noise ratio 10 : 1 according to (ICH, 2005)

Results and Discussion
System suitability
Column efficiency for selenium peak was 7500 plates and for nano selenium was 9700 plates as Theoretical plates (N) according to USP is =≥2000. Peak asymmetry factor (As) was 1.158 for selenium and for nano selenium was 0.95 which considered fronting as As was less than 1. Tailing factor as USP coefficient (T = ≤2.0)of the peak symmetry was 1.03 and 0.99 for selenium and nano selenium, respectively.
Precision
Precision of the developed method was determined based on inter and intra-day precisions. Results are presented in Table (1). The method was found to be precise since the RSD values for both inter-day and intra-day precision was below 1.0

2.a. Intraday Precision

Selenium			Nano-selenium	
Sr. no.	**Concentration (µg/ml)**	**Peak area (In the same day)**	**Concentration (µg/ml)**	**Peak area (In the same day)**
1	0.02	40.84	0.08	38.9
2	0.02	40.77	0.08	38.029
3	0.02	40.4	0.08	38.3111
4	0.02	40.59	0.08	38.205
5	0.02	40.7	0.08	38.57778
6	0.02	40.34	0.08	38.6127
Mean		40.64		38.43926
SD		0.2		0.316596
RSD%		0.5		0.823628

2.b. Inter day precision value:

Selenium			Nano-selenium	
Sr. no.	**Concentration (µg/ml)**	**Peak area (In six days)**	**Concentration (µg/ml)**	**Peak area (In six days)**
1	0.02	40.014	0.08	38.3098
2	0.02	39.887	0.08	38.72
3	0.02	39.942	0.08	38.804
4	0.02	39.975	0.08	38.88
5	0.02	40.005	0.08	37.943
6	0.02	40.42	0.08	38.775
Mean		40.041		38.57197
SD		0.192		0.36775
RSD%		0.478		0.95341

linearity and range

The matrix-matched calibration standards were prepared by spiking the blank material with the same spiking solution as for samples for validation with variable volumes. Standard curves were constructed by spiking of eight blank muscle and liver tissue broiler samples with various volumes of selenium and nanoselenium stock solution to yield a concentration range of 0.005, 0.02, 0.04, 0.08, 1.0, 0.25, 0.5, 1 and 2.5 µg/ml and 0.01, 0.02, 0.04, 0.08, 0.1, 0.25, 0.5, 1 and 2.5µg/ml, respectively.

The Standard curves were shown linearity from 0.005 to 2.5µg/ml for selenium standard in solvent **fig. (1-b),** muscle tissue samples **fig. (1-d)** and in liver tissue **fig. (1-f)**. Meanwhile, The linearity from 0.01 to 2.5 µg/ml

for nano-selenium standard in solvent **fig (1.a)**, blank muscle samples **figure (1-c)** and in liver tissues **figure (1-e).**

The linearity of peak area responses versus concentrations was demonstrated by linear least square regression analysis. The linear regression equations and correlation coefficient (r^2) were illustrated in **figure1 (a, b, c, d, e & f)**.

Linearity values and peaks area were shown in **table (3).** Analytical performance parameters were shown in **table (4).**

Table (3). linearity of Selenium and nano-selenium standard in solvent:

Selenium				Nano-selenium			
RT	**Level**	**concentration (µg/ml)**	**Peak area**	**RT**	**Level**	**Conc.(µg/ml)**	**Peak area**
3.765	1	0.005	9.8	7.073	1	0.01	2.741
	2	0.02	40.899		2	0.02	8.1
	3	0.04	80.5		3	0.04	18.05
	4	0.08	161.2		4	0.08	39.9
	5	0.1	207.99		5	0.1	50.022
	6	0.25	518.85		6	0.25	128.14
	7	0.5	1001.8		7	0.5	260.42
	8	1	2012.6		8	1	524.84
	9	2.5	5113		9	2.5	1272.5

*RT: Retention Time

Table (4). Analytical performance parameters

Parameters	**Selenium**	**Nano-selenium**
	Value	
Linearity range	0.005-2.5 µg/ml	0.01- 2.5 µg/ml
Correlation coefficient(r^2)	0.9999	0.9998
Slope (a)	2042.1	511.04
Intercept (b)	-0.4839	0.7519
Regression equation	y= 2042.1x–0.4839	Y=511.04x + 0.7519

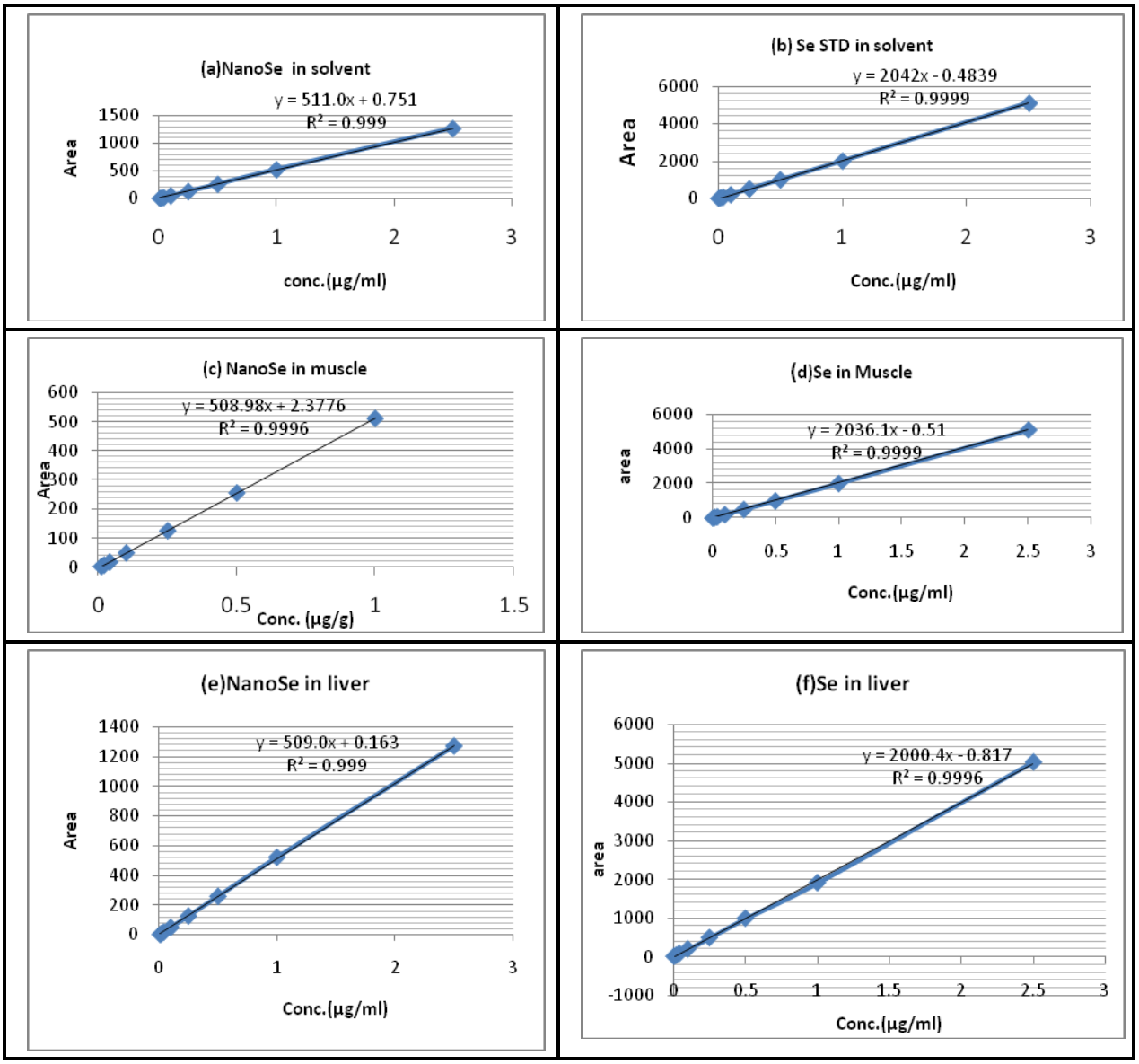

Figure (1). Matrix-matched calibration plots for nano-selenium (a) and Selenium (b) in solvent , chicken meat (c,d) and Liver tissues (e,f).

Specificity

The chromatograms compared to know that there is no excipient compound interference between peaks of the pure standard and peaks of spiked tissue (muscle and liver) samples. Figure 2.

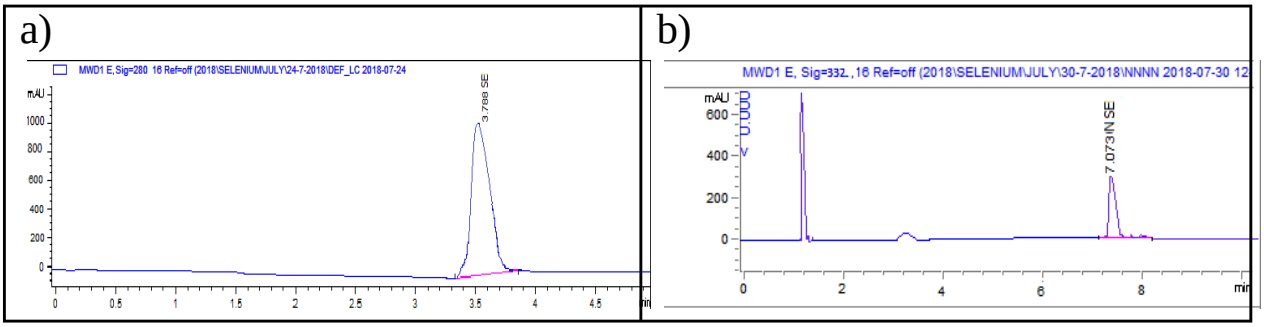

Figure (2). Chromatogram of spiked blank muscle sample with (a) selenium and (b) nano-selenium (0.1µg/ml)

Accuracy and Recovery

Spiked samples will be prepared at three concentrations of 50, 100 and 150 % which represented by 0.02, 0.04, and 0.08 µg/ml respectively. Each spiked sample was prepared in triplicate at each level and injected. The absolute recovery of selenium and nano-selenium were shown in table (5).

Table (5). Recovery & Accuracy studies

(a) Selenium

Concentration level (µg/ml)	Found Conc. (µg/ml)	Recovery %	Mean ± SD	RSD%	Average % Recovery (Accuracy) ± SD
0.02 (50%)	0.02004	100.2007	0.02012± 0.000074	0.36973	100.601±0.372
	0.02013	100.6664			
	0.02019	100.936			
0.04 (100%)	0.039657	99.14292	0.03979± 0.00037	0.92105	99.47754±0.92
	0.03951	98.77565			
	0.04021	100.5141			
0.06 (150%)	0.059098	98.49656	0.059415± 0.000328	0.553214	99.02434±0.54782
	0.059392	98.98626			
	0.059754	99.59021			

(b) Nano-selenium

Concentration level (µg/ml)	Found Conc. (µg/ml)	Recovery %	Mean±SD	RSD%	Average % Recovery (Accuracy) ± SD
0.02 (50%)	0.02005	100.26517	0.0198377± 0.0001905	0.96	99.1887±0.952
	0.01969	98.45632			
	0.019769	98.84461			
0.04 (100%)	0.03975	99.37874	0.039723±0.00 0309	0.77731	99.30771±0.772
	0.040017	100.04167			
	0.0394011	98.502727			
0.06 (150%)	0.0597341	99.5567846	0.0598793±0.0 0032	0.53337	99.7988±0.53231
	0.05965831	99.43051241			
	0.0602451	100.4091219			

Robustness:

The results of robustness indicated that changing the mobile phase composition and changing the pH of citrate buffer of mobile phase had slight effect on the chromatographic behavior of nano-selenium. However, changing the column had no significant effect, also; changing in the mobile phase composition and wavelength had a slight effect. The RSD % of robustness testing under different altered conditions are given in Table (6) and (7), indicating that the current methods were robust.

Table (6). Results of Robustness study (Nano-selenium concentration, 0.08 µg/ mL, n=3)

Chromatographic parameter	Modification	Peak Area Precision (n=3)	Mean area ± SD	RSD%
Change in Mobile phase composition	10mM citric acid monohydrate: Methanol **15:85** 17:83 13:87	 38.44895 37.193573 37.678667	37.77373± 0.63306	1.675938
pH of citrate buffer of mobile phase	2.3 **2.5** 2.7	37.7513 38.7287 39.6	38.6933±0.92487	2.3903
Change in Column	**C8** C18	38.2125 37.70291	37.95771±0.360332	0.949299

Table (7). Results of Robustness study (Selenium concentration, 0.02 µg/ mL, n=3)

Chromatographic parameter	Modification	Peak Area Precision (n=3)	Mean area ± SD	RSD%
Change in Mobile phase composition	Methanol: Water **100% methanol** 93:7	39.78451 38.40466	39.09459±0.975701	2.495744
Change in Wavelength(nm)	**280** 277 283	39.87977 38.72295 39.3635	39.32207± 0.579525	1.47379

Limit of detection and limit of quantification

LOD and LOQ were calculated using the following equations designated by International Conference on Harmonization (ICH) guidelines.

$LOD = 3.3 \times \sigma/S$

$LOQ = 10 \times \sigma/S$

Where, σ is the standard deviation of intercept S is slope of the calibration curve. So, LOD of Selenium was 0.008657 µg/ ml and LOQ was 0.026234µg/ml. On the other hand; LOD of nano-selenium was 0.014032 µg/ ml and LOQ was 0.046775µg/ml.

Discussion

The development of HPLC methods for detection and quantification of analytes is very important in analytical researches for achieving the quality control. Despite all previous experimental studies for development and validation of analytical methods for determination of selenium and nano-selenium in chicken tissues with different detectors as **(Li *et al.*, 1999 and Pedersen& Larsen, 1997)** which carried out the separation of selenium compounds by HPLC on an ESA Anion III anion-exchange column and on-line selenium-specific detection was carried out with an inductively coupled plasma mass spectrometer (ICP-MS) or a flame atomic absorption spectrometer (FAAS), these methods achieved the detection of selenium compounds by RP-HPLC through following the validation parameters such as accuracy, precision, robustness, limit of detection (LOD) and limit of quantification (LOQ) according to

ICH guidelines. The developed methods were reliable, simple, sensitive, precise, accurate and economic as using an UV detector. Meanwhile, other developed methodsas **(Nakagawa *et al.*, 1989 and Tanaka *et al.*, 1988)** which used a fluorometric detector in detection of selenium compounds. The developed method, using simple HPLC grade solvents (methanol and water), had a short retention time and high peak symmetry. The method developed was validated successfully. The selected mobile phase system gave a single sharp peak for each selenium and nano-selenium without interfering peaks. The retention time (RT) of was 3.765 min and 7.073 with a tailing factor 1.03 and 0.99 for selenium and nano selenium, respectively. The method was linear in the concentration range of 0.005 – 2.5µg/ml and 0.01- 2.5µg/ml for selenium and nano selenium, respectively, and the calibration curves showed good correlation between concentration and peak area.

Conclusion

In this work, we have presented a new method for the extraction and pre-concentration of selenium and Nano- selenium as standard in solvent and in chicken tissues (muscle and liver). The proposed methods were based on the use of methanol (organic solvent) in selenium validated method to make a compound with selenium which called dimethylsiloxane (SiOcH3).

On the other hand; we used DAB (2,3-diaminobenzene) reagent to react with nano-selenium for synthesis of 2,1,3 benzoselenadiazole (BSD). These two compounds allowed the extraction and pre-concentration of selenium and nano-selenium after the derivatization step, and so could be detected by Vis-UV HPLC. The developed and validated Vis-UV HPLC methods were found to be more economical. The result of analysis of formulation and recovery studies obtained by HPLC method were statistically validated and high percentage of recovery studies suggest that the developed methods were free from interference of excipients used in formulation. The Vis-UV HPLC methods were statistically validated in terms of accuracy, precision, linearity and reproducibility. Hence above methods can be employed in laboratories to estimate selenium and nano-selenium in chicken tissues (muscle and liver).

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